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Title

Growth media in anaerobic fermentative processes: the underestimated potential of thermophilic fermentation and anaerobic digestion

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Abstract

Fermentation and anaerobic digestion of organic waste and wastewater is broadly studied and applied. Despite widely available results and data for these processes, comparison of the generated results in literature is difficult. Not only due to the used variety of process conditions, but also because of the many different growth media that are used. Composition of growth media can influence biogas production (rates) and lead to process instability during anaerobic digestion. To be able to compare results of the different studies reported, and to ensure nutrient limitation is not influencing observations ascribed to process dynamics and/or reaction kinetics, a standard protocol for creating a defined growth medium for anaerobic digestion and mixed culture fermentation is proposed. This paper explains the role(s) of the different macro- and micronutrients, as well as the choices for a growth medium formulation strategy. In addition, the differences in nutrient requirements between mesophilic and thermophilic systems are discussed as well as the importance of specific trace metals regarding specific conversion routes and the possible supplementary requirement of vitamins. The paper will also give some insight into the bio-availability and toxicity of trace metals. A remarkable finding is that mesophilic and thermophilic enzymes are quite comparable at their optimum temperatures. This has consequences for the trace metal requirements of thermophiles under certain conditions. Under non-limiting conditions, the trace metal requirement of thermophilic systems is about 3 times higher than for mesophilic systems.

Keywords

Nutrient, trace metal, volatile fatty acid, fermentation, anaerobic digestion, mesophilic, thermophilic, enzyme, hydrogen, biogas

Contents

1 Introduction

Anaerobic digestion or fermentation of organic waste(water) is widely applied and studied. The most commonly pursued anaerobic mixed culture end products are methane (CH_4), hydrogen (H_2) and volatile fatty acids (VFA) (Gujer and Zehnder, 1983; Henze, 2008). Due to the numerous studies on anaerobic digestion and fermentation, results and data are widely available for these processes. However, comparison of the generated results in literature is difficult, not only due to the used variety of process conditions, but also because of the different growth media that are used (Angelidaki et al., 2009; Cheong et al., 2007; Fang and Liu, 2002; Hawkes et al., 2007; Pobeheim et al., 2010; Temudo et al., 2007). Different nutrient media can highly influence research results and so, ideally, the composition of nutrient media should be the same in order to compare the different experiments reported in literature (Bourriaud et al., 2005; Fang and Liu, 2002, 2002; Muñoz Sierra et al., 2017; Turton et al., 1983).

In general, a growth medium consists of so called macro- and micronutrients, in which the concentration of each component depends on the required quantity. The elements carbon, oxygen, hydrogen, nitrogen, phosphorus, potassium, sulphur, calcium and magnesium are categorized as macronutrients since relatively large quantities are required, particularly, for cellular growth. All other nutrients, such as the trace elements iron, cobalt and nickel, and vitamins, are mainly used for enzyme or co-factor production. They are needed in much lower quantities and are therefore categorized as micronutrients.

A shortage of trace metals can result in lower biogas production (rates) and process instability during anaerobic digestion processes (Fermoso et al., 2008; Pobeheim et al., 2010). Speece (1988) suggested, based on a survey of 30 municipal anaerobic sludge digesters, which included conducting activity assays, that 17 of the 30 digesters were trace metal limited with regard to acetate and propionate degradation rates. The rate of methane production from acetate (8 digesters) and propionate (9 digesters) was stimulated by addition of the trace metals iron, nickel or cobalt (Speece, 1988). Furthermore, the addition of trace metals like iron, nickel, cobalt, molybdenum, selenium and tungsten have been reported to increase acetate, propionate and H_2/CO_2 conversion and conversion rates (Banks et al., 2012; Boonyakitsombut et al., 2002; Espinosa, 1995; Moestedt et al., 2015; Ortner et al., 2015; Plugge et al., 2009; Speece et al., 1983). The observed stimulation of the anaerobic digestion process by the addition of metals was probably caused by a low amount of readily available trace metals in the inoculum and substrate (Solis et al., 2002; Zandvoort et al., 2006).

To compare reported results of the different studies and to make sure nutrient limitation is not influencing observations ascribed to process dynamics and/or reaction kinetics, a standard protocol for creating a defined growth medium for anaerobic mixed culture processes is urgently required. This paper explains the role(s) of the different macro- and micronutrients, as well as the choices for a growth medium formulation strategy. In addition, the differences in nutrient requirements between mesophilic and thermophilic systems are discussed as well as the importance of specific trace metals regarding specific conversion routes and the possible supplementary requirement of vitamins. Finally, the paper provides some information on the bio-availability and toxicity of trace metals. The overall focus in this paper is on systems with trace metal limitation and maximized growth rates.

2 Roles of the different macro- and micronutrients

A growth medium is a medium in which all the necessary elements (or nutrients) for the growth of cells are present. The necessary elements are elements that are used for energy production, growth, and maintaining cell functions by organisms. In tables 1 and 2, these elements are categorized into macronutrients and micronutrients, and a description of each element is provided, including their role(s) in cellular function, anaerobic digestion and fermentation processes.

3 Growth medium formulation strategy

Many studies on methanogenesis, anaerobic digestion (AD) and mixed culture fermentation have been conducted with synthetic wastewaters, to which growth nutrients must be added. Also many industrial wastewaters lack nutrients and require growth nutrients supplementation prior to anaerobic treatment. Growth media should contain both macro- and micronutrients. However, because of the different roles of macro- and micronutrients in growing (mixed) cultures, the design of a macronutrient medium requires a different strategy than a micronutrient medium. These strategies will be explained in the following sections.

3.1 Macronutrients

Macronutrients (C, N, P, K, Na, S, Ca and Mg) are the elements which are mainly used for anabolism Since substrate loading rate and the biomass yield determine the biomass formation, required quantities depend on these two factors during the anaerobic conversion process. In table 3, the average biomass yields of the different phases in anaerobic fermentation are given for both mesophilic and thermophilic conditions. The specific composition of the required macronutrient medium depends on the elemental cell composition of the growing biomass, which is given in table 4.
If the macronutrient requirements were expressed in mg/g TSS instead of mg/g VSS, an average VSS/TSS ratio of 0.85 was used for recalculation (Metcalf, 2005; Takashima et al., 2011). Furthermore, it is assumed that the biomass composition of acidifiers, acetogens and methanogens, in mesophilic and thermophilic systems, is the same.

The biomass yields in table 3 are maximum yields. Observed yields can be lower at increased solids retention times (SRTs), because of an increase in maintenance energy, cell lysis and possible predation effects (Metcalf and Eddy, 2003).

The required amount of macronutrients can be calculated using the following equation:

$$C_E = COD_{bio-influent} * Yield * E_{Biomass} \quad \text{(eq 1)}$$

C_E = Concentration of target element (mgE/l)
$COD_{bio\text{-}influent}$ = Biodegradable Chemical Oxygen Demand in the influent (g/l)
Yield = Biomass Yield (g VSS/g COD_{bio})
$E_{Biomass}$ = Element concentration in biomass (mg Element/g VSS)

In order to avoid macronutrient limitations that could influence the fermentation pattern (Carlsson and Griffith, 1974), it is proposed to use the highest theoretical maximum biomass yield, regardless of the applied SRT, when calculating the initial macronutrient dosage required. After reaching steady state in the process, the observed yield could be determined and the composition of the nutrient solution could be changed accordingly, to minimize overdosing with macronutrients.

Two elements in macronutrients should be specially considered: carbon and sodium.
For carbon dosage no value is proposed. This is because, in many cases, carbon originates directly from the dosed and converted substrate, so “additional” supply is not required. However, in some cases, CO_2 is required for product and/or biomass formation, as is the case for e.g. hydrogenotrophic methanogens (Metcalf, 2005). In addition, the acidifier *Selenomonas ruminantium* takes up CO_2 to form products (Linehan et al., 1978; Paynter and Elsden, 1970) and *Clostridium kluyveri* specifically

needs CO_2 to form biomass (Tomlinson and Barker, 1954; Tomlinson, 1954a, 1954b). In these cases an additional carbon supply could be considered.

Sodium is an element which is mainly used as a counter ion for cellular buffers, electrolytes and DNA, and as a solute for exchange and transport processes and energy generation (Maret and Wedd, 2014). In general, sodium concentrations of 100-200 mg/l are beneficial for mesophilic anaerobes (McCarty, 1964). There are however, bacteria and methanogens, like halophilic methanogens, which require a high(er) concentration of sodium (Jarrell and Kalmokoff, 1988; Maret and Wedd, 2014; Scherer et al., 1983).

3.2 Micronutrients

Micronutrients are, by definition, essential nutrients or trace elements that are required by an organism in minute amounts. The trace elements are mainly used for the production and functioning of enzymes and co-factors. In table 2 an overview is given of which micronutrients are required for which enzymes.

The (initial) composition of a chemically defined medium is usually based on the cellular composition and desired cell concentration of the microbe of interest and therefore similar to the approachto determine the composition of a macronutrients medium (Greasham and Herber, 1997; White et al., 1990; Zhang and Greasham, 1999). However, when calculating the required amounts of trace elements for AD and mixed culture fermentations this is probably not the most optimised approach, since the cellular composition of all different micro-organisms is usually not known and only overall biomass compositions can be determined. Also, the required amount of maintenance energy of the different micro-organisms can vary depending on operational conditions. For example, maintenance energy requirement will be high(er) under extreme pH conditions, at temperatures higher than the optimal growth temperature of the organisms or when high undissociated volatile fatty acid (VFA) concentrations are present (Röling and Van Verseveld, 1997; Russell, 1992). The latter increase in maintenance energy might be caused by the diffusion of undissociated VFA over the cell wall, where after they will dissociate inside the cell because of more neutral pH values inside the cell (Russell, 1992; Russell and Diez-Gonzalez, 1997). This would lead to increased intracellular protons and dissociated VFA concentrations inside the cell. In order to prevent intracellular pH decrease and VFA accumulation, protons and VFA will be actively transported out (Russell, 1992; Russell and Diez-Gonzalez, 1997; Tijhuis et al., 1993), leading to an increased maintenance energy requirement and thus to reduced growth (Russell, 1992; Russell and Diez-Gonzalez, 1997).

However, to our knowledge, the effect on enzyme production under suboptimal growth conditions is unknown. The enzyme production could be either higher or lower compared to production under optimal conditions. Therefore, when determining the required amount of trace elements, it is assumed that the enzyme production under both optimal and suboptimal growth conditions is similar. This implies the same trace element requirement under both optimal and suboptimal growth conditions.

Because of this assumption the required amount of trace elements should be based on the biodegradable COD concentration of the waste stream to be treated and not on the biomass yield.

Consequently, the amount of required trace elements can be estimated using the following equation:

$$C_E = COD_{bio-influent} * E \qquad \text{(eq 2)}$$

C_E = Concentration of Element (µg/l)
$COD_{bio\text{-}influent}$ = Biodegradable COD in influent (g/l)
E = required Element amount (µg Element/g COD_{bio})

In table 5 and 6 a summary of the proposed trace element requirements for mesophilic acidogenic and acetogenic/methanogenic systems is provided. Values are given in micrograms of micronutrients per gram COD_{bio} in the system and were determined via a stepwise approach. First, if available, literature data regarding the mixed culture was collected and inventoried. If data regarding mixed cultures was

not available, data regarding specific key-organisms, which are present in many cases, was sourced. If no data for mesophilic acidogens could be found, the average value of the methanogens was used, multiplied by 5, because the trace element requirement of acidogens is higher than for methanogens (comparison of iron, nickel, cobalt and zinc, table 5 and 6), and hereby preventing limitation.

For some trace elements, some special behaviour was found and is highlighted below.
In many cases molybdenum (Mo) and tungsten (W) are acting as antagonists for each other (Andreesen and Ljungdahl, 1973; Ljungdahl and Andreesen, 1975; Plugge et al., 2009; Zellner et al., 1987).

Copper is an element which is commonly required by aerobic bacteria and aerobic archaea, but only required by a small number of anaerobic and facultative bacteria, and facultative anaerobic archaea (Ridge et al., 2008). The only information that could be found in literature regarding the copper requirement of these organisms is presented in table 6. This is in contradiction with the findings of Ridge et al. (2008), who couldn't establish a copper requirement for the anaerobic archaea they tested. Further research is needed to obtain information regarding the possible copper requirements of acidifiers and methanogens. Since no information in literature could be found regarding the requirement of copper for acidogens, the same value as for methanogens has been used. For the cobalt requirement of mesophilic methanogens two different values are mentioned in literature. The higher value of 25 µg/g $COD_{bio\text{-}influent}$ should only be used in the case of direct conversion of methanol into methane (Florencio et al., 1994).

The order for the different required concentrations of trace elements for methanogens is:
Fe>Ni/Co>Mo (and/or) W > Zn>Cu/Mn according to Ferry (2010) and Glass and Orphan (2012).
Table 6 follows this order, with the exceptions of zinc and copper.

To our knowledge an order of trace element concentrations for acidifiers doesn't exist.

In case of biomass retention in an anaerobic reactor (SRT>HRT), intracellular enzymes also retain in the reactor, implying that the trace metal requirement for intracellular enzymes decreases. Extracellular enzymes, however, can be bound to the (particulate) substrate or free moving in the liquid. In case the substrate is also retained in the reactor, the trace metal requirement would decrease, but if the substrate is washed out due to the low HRT, the trace metal requirement is determined by this HRT and will not change due to biomass retention.

4 Enzyme activity at different temperatures and its influence on the nutrient requirement in thermophilic and mesophilic systems

Different operational temperatures are used in anaerobic fermentation and digestion systems; optimal mesophilic conditions vary from 30 to 40ºC, while moderate thermophilic conditions have an optimal operational temperature range of 50 to 60ºC (Madigan et al., 1997; Metcalf, 2005). A frequently cited advantage of thermophilic systems is the possible higher conversion rate, or higher loading rate that can be applied, in comparison to mesophilic systems (Ahn, 2000). The higher conversion rates could be caused by higher growth rates; thermophilic methanogens have a growth rate which is, in general, a factor of 2 to 3 times higher when compared to mesophilic homologues methanogens (Borja et al., 1995; Lier, 1995; Speece, 1996). Taking into account that the maintenance energy requirement of thermophilic bacteria and archaea at their optimal growth temperature is higher than the maintenance energy requirement of mesophilic bacteria and archaea at their optimal temperature (Borja et al., 1995; Lier, 1995), then the substrate conversion rates in thermophilic systems could possibly increase even more than the increase of the growth rate. For example, Cecchi et al. (1991) mentioned that the first order kinetic constant of substrate utilization of municipal solid waste is four times larger under thermophilic conditions than under mesophilic conditions. The higher conversion rates of particulate substrates under thermophilic conditions could however, also be caused by other factors; like increased access to the substrate, higher solubility of the substrate, lower liquid viscosity (better mixing) or higher diffusion rates of soluble compounds.

One would expect that at higher temperatures the specific conversion rates of enzymes would also be higher. However, the specific rates of thermophilic enzymes and their mesophilic homologue counterparts are often described as being similar at their respective temperature optima (Amelunxen and Murdock, 1978; Danson et al., 1996; Fágáin, 1995; Huber and Bennett, 1983; Varley and Pain, 1991; Wolf-Watz et al., 2004; Zavodszky et al., 1998). Furthermore, the catalytic efficiencies of thermophilic, mesophilic, and psychrophilic enzymes appear to be similar at their respective operational temperatures (Coquelle et al., 2007; Georlette et al., 2003). Enzyme activity is found to be dependent on enzyme conformational flexibility (Artymiuk et al., 1979; D'Auria et al., 1999; Frauenfelder et al., 1979; Huber and Bennett, 1983; Lipscomb, 1970). Conformational flexibility is the ease with which the shape of an enzyme can be altered. Usually it is a modification to the tertiary structure of an enzyme as a consequence of changes in pH, temperature, ionic strength of the environment, or the binding of a substrate to an enzyme (Huber, 1979). This conformational flexibility of mesophilic and thermophilic enzymes is found to be similar at their respective temperature optima (Daniel et al., 1996; Jaenicke, 1996; Zavodszky et al., 1998). Furthermore, Feller (2010) observed from crystal structures of extremophilic enzymes that all reactive side chains as well as most side chains pointing towards the catalytic cavity are strictly conserved, compared to mesophilic enzymes. Crystal structures of enzymes are the structures of an enzyme at the atomic level in different conformational positions (Huber, 1979). The three-dimensional structures of mesophilic and thermophilic enzymes appear to be superposable (Auerbach et al., 1998; Chi et al., 1999; Hopfner et al., 1999; Isupov et al., 1999; Maes et al., 1999; Russell et al., 1997; Tahirov et al., 1998). This all suggests that the overall catalytic mechanism, reaction pathway and enzymatic properties of the relevant different enzymes are similar under mesophilic and thermophilic temperature conditions (Bauer and Kelly, 1998; Jaenicke, 1991; Ljungdahl, 1979; Vieille et al., 1995; Wrba et al., 1990; Zwickl et al., 1990).

In addition to having equivalent conversion rates and catalytic routes, thermophilic enzymes also (roughly) double their rate with every 10°C increase (Q_{10} of 2) just like mesophilic enzymes (Elias et al., 2014). This underlines the impression that mesophilic and thermophilic enzymes are fully comparable at their optimum temperatures; regarding structure, catalytic route and thermo-sensitivity. This would imply that the increased conversion rates found in thermophilic systems, compared to mesophilic systems, is not due to increased conversion rate per enzyme but is possibly caused by a higher concentration of enzymes in these systems. Very recently, Ghasimi et al. (2015) found strong indications of higher enzyme concentrations in thermophilic systems in comparison to mesophilic systems. Further research is needed to confirm this.

If the enzyme concentration indeed is a factor of 2 to 3 times higher in a thermophilic system compared to a mesophilic system, the amount of micronutrients that are needed for the good functioning of a thermophilic system should also be 2 to 3 times higher compared to a similar mesophilic system. This reasoning is in line with several research results. For example, Takashima et al. (2011) investigated the iron, nickel, zinc and cobalt requirements of a mesophilic and thermophilic system during the conversion of glucose to methane and found that the requirements for the thermophilic system were 2.2 to 7.8 times higher than those for the mesophilic system. Uemura (2010) also concluded that thermophilic anaerobic digestion has a higher trace element requirement than mesophilic anaerobic digestion. Zellner and Winter (1987) investigated the tungsten requirements for hydrogenotrophic methanogens and the results showed that the thermophilic methanogens had a tungsten requirement that was at least 2.5 times higher than the tungsten requirements of the mesophilic methanogens. *Clostridium Pastuerianum* and *Clostridium Welchii* need respectively 0.11 (Schönheit et al., 1979a) and 0.12-0.16 mg Fe/g $COD_{substrate}$ (Pappenheimer and Shaskan, 1944) for optimal growth, which is in line with the amount that Takashima et al. (2011) found for the mesophilic acidogenic phase of digestion (0.177 mg Fe/g $COD_{substrate}$). According to Nishio et al. (1991) the optimum iron dosage for the thermophilic *Clostridium Thermoaceticum* is 0.28-0.84 mg Fe/g $COD_{substrate}$, which is about 2.5-5 times higher compared to mesophilic Clostridia. This is also in line with the results of Takashima et al. (2011), who found an iron demand for thermophilic systems that was 2.25 times higher than for mesophilic systems.

From the preceding information it can be concluded that mesophilic and thermophilic enzymes are similar in catalytic route, structure and enzymatic activity and that the trace element requirements for both mesophilic and thermophilic systems will increase if sludge loading rates (kg COD/kg VSS.day) increase (according to equation 2).

The proposed required amount of trace elements for thermophilic acidogenic and acetogenic/methanogenic systems are provided in tables 7 and 8. Values are given in microgram of micronutrient per gram biodegradable COD in the system. These values were determined following the same stepwise approach as for the mesophiles. If no data for thermophilic acidogens or methanogens could be found, the proposed amount for mesophilic acidogens or methanogens was used, multiplied by a factor of 3. For copper the same value as for the mesophilic systems was used without multiplication, because copper is very toxic (Lin and Chen, 1997; Sanchez et al., 1996).

Very little is known regarding the boron requirements for the growth of anaerobic bacteria and for Archaea these requirements have yet to be evaluated (Kabay et al., 2015). The growth of *Saccharomyces cerevisiae* is however, stimulated by boron (Bennett et al., 1999). Boron is in some cases also used for bacterial quorum-sensing (Chen et al., 2002). The addition of boron to the nutrient solution is by the experimenter's discretion.

5 Specific conversion pathways depend on the presence of specific trace metals

The proposed approach (above) to define the micronutrient solution is very general. It should be noted that different fermentation pathways may require a different composition of trace elements in solution. The section below describes some deviations from the general approach. Likely, the overview is far from complete, since many possible fermentation routes and their specific trace element requirements have not been reported in literature or are not yet known. If the specific described route is the dominant or preferred route, the required trace elements should be present in sufficient bio-available quantities. This means they should either be in solution, dissolving at a non-limiting rate, or be liberated, in sufficient quantities, during bio-degradation of the substrate.

The AD process can be interpreted as a sequence of four different microbial conversion steps; hydrolysis, acidogenesis, acetogenesis and finally methanogenesis (Metcalf, 2005). The specific conversion pathways in each of these steps depends on the presence of specific trace elements as illustrated below.

5.1 Hydrolysis

Hydrolysis is the process in which exo-enzymes and/or membrane bound enzymes convert complex particulate compounds into less complex dissolved compounds. The most important hydrolytic enzymes are cellulases, amylases, proteases and lipases and are produced by acidogenic bacteria. The working principle of these enzymes is discussed in depth in several papers (Brahmachari et al., 2017; Rao et al., 1998; Schwarz, 2001). The functioning of some of these hydrolytic enzymes also depends on trace metals. For cellulases this is calcium, which is amongst others, important for the folding of the cellulases (Brahmachari et al., 2017; Lytle et al., 2000; Schwarz, 2001). Amylases need in many cases calcium for their activity, structural integrity and stability (Brahmachari et al., 2017). For some proteases zinc, cobalt, manganese and/or calcium are essential, whereby the first three can stimulate the activity of amylases(Bertini and Luchinat, 1994; Holmes and Matthews, 1981; Latt et al., 1969; Jisha et al., 2013; Rao et al., 1998). Lipases generally don't require metals to function, but the lipase activity is in many cases stimulated by calcium (Gupta et al., 2004).

To our knowledge there is no information present regarding the amount of produced hydrolytic enzymes by different bacteria during mixed culture fermentation or digestion, and thus the trace metals requirement regarding hydrolytic enzyme production is also difficult to estimate: further research on this topic is needed.

5.2 Acidogenesis

Acidogenesis is the step in which organic compounds are mainly converted into VFAs during microbiological processes. The conversion of organic compounds into acetate and butyrate is accompanied by hydrogen formation. This involves hydrogenases and therefore the trace elements iron, nickel, zinc, and selenium are important (see table 2).
In some cases, such as for some acetate-propionate producing *Selenomonas* strains, there is no production of hydrogen during fermentation (Scheifinger et al., 1975). Nonetheless, hydrogenases are involved in the production of propionate (Henderson, 1980). This means that the trace elements iron, nickel, zinc and selenium are still important. In addition to these elements, in some cases vitamin B_{12} is also required for the production of propionate (Chen and Wolin, 1981; 1992). Vitamin B_{12} is a cobalt containing vitamin. The bacteria *Selenomonas ruminantium* and *Megasphaera elsdenii* can produce vitamin B_{12} independently and therefore, only need the supplementation of cobalt (Dryden et al., 1962a). Bacterium *Prevotella ruminicola*, however, is not capable of producing (enough) vitamin B_{12} for itself (Chen and Wolin, 1981). In this case, the vitamin needs to be added or produced by other bacteria, e.g. *Selenomonas ruminantium*, in the culture. It has been observed that when insufficient vitamin B_{12} is present succinate is produced instead of propionate (Chen and Wolin, 1981; Strobel, 1992). Also other acidifiers have a vitamin or amino-acid requirement like several cellulolytic acidogens (Scott and Dehority, 1965) and *Megaspheara elsdenii* (Miura et al., 1980). An overview of several acidifiers having a vitamin or amino-acid requirement can be found in Hobson and Stewart (1997).

5.3 Acetogenesis

Acetogenesis comprises of microbiological processes where the products of fermentative bacteria are converted into acetate, hydrogen and carbon dioxide. To our knowledge only (part of) the trace element requirement of the syntrophic propionate oxidizer *Syntrophobacter fumaroxidas* is known, which requires iron, selenium and tungsten to produce formate dehydrogenases (de Bok et al., 2003), while analysis of other trace elements needed for acetogenesis is lacking: Therefore, further research is needed to obtain a better understanding of their trace metal requirements.

5.4 Methanogenesis

Methane can be formed via three different pathways; namely the hydrogenotrophic, methylotrophic and aceticlastic pathway (Glass and Orphan, 2012). The different pathways are explained below.

Hydrogenotrophic pathway
The formation of methane from H_2/CO_2 and formate is called hydrogenotrophic methanogenesis. Important trace elements for the conversion of H_2/CO_2 and formate to methane are iron, nickel, cobalt, selenium, molybdenum and tungsten (Banks et al., 2012; Espinosa, 1995; Jiang, 2006; Kim et al., 2002; Osuna et al., 2003; Öztürk, 1991; Plugge et al., 2009; Worm et al., 2009). This hydrogenotrophic pathway is sometimes found to be the dominant methane formation pathway, for example, in digesters with high ammonium concentrations (Angelidaki and Ahring, 1994; Demirel and Scherer, 2008; Gallert et al., 1998; Koster and Lettinga, 1984; Zinder, 1990).

Methylotrophic pathway
The direct formation of methane from methanol is called the methylotrophic pathway. Important trace elements for the conversion of methanol to methane are iron, zinc, nickel and especially cobalt (Fermoso et al., 2008; Florencio et al., 1994; Nishio et al., 1992; Zandvoort et al., 2002a; Zandvoort et al., 2002b; Zandvoort et al., 2004). For the direct conversion of methanol to methane the cobalt concentration should be in the range of 0.5-2 µM. However, at higher cobalt concentrations methanol degradation may proceed via acetate formation and subsequent aceticlastic methanogenesis, or via oxidation to CO_2 and H_2 followed by hydrogenotrophic methanogenesis, given that the H_2 partial pressure is low enough (Florencio et al., 1994; Jiang, 2006)

Aceticlastic pathway

The formation of methane from acetate by splitting the acetate molecule is called the aceticlastic pathway. Important trace elements for this conversion are iron, nickel, cobalt and zinc (Boonyakitsombut et al., 2002).
In some cases selenium has a positive influence on the acetate conversion to methane (Banks et al., 2012). Acetate, however, can also be anaerobically oxidised to H_2/CO_2 and then to methane (Cord-Ruwisch et al., 1988). In that case, the trace elements essential for the hydrogenotrophic pathway should be present in sufficient bio-available quantities. Unfortunately, the required trace elements for acetate oxidation are still unknown.

6 Vitamins

Many methanogenic archaea need vitamins if they are mono-cultured or cultured without acidifiers (Jarrell and Kalmokoff, 1988; Speece and McCarty, 1964; Wolin et al., 1963). Also some (cellulolytic) acidifiers and lactic bacteria require vitamins for growth (Bornstein and Barker, 1948; Mulder, 1990; Pritchard and Coolbear, 1993). However, it has been shown that bacteria can synthesize certain vitamins (Bechdel et al., 1928; Dryden et al., 1962b; Hill, 1997; LeBlanc et al., 2013; Scott and Dehority, 1965; Ye et al., 1996). Mixed culture fermentations often do not need additional vitamin supplementation to function. There is apparently a(n) (inter)dependency of bacteria and archaea regarding vitamins. The latter was already hypothesized by Thompson (1942).

Most anaerobic mixed cultures are self-sustaining with regard to vitamins and amino acids and a simple medium is sufficient (Mulder, 1990; Speece and McCarty, 1964). However, in some cases, e.g. when treating specific industrial wastewaters or in pure culture studies, the production of sufficient vitamins to sustain the biological processes are lacking (Bornstein and Barker, 1948; Jarrell and Kalmokoff, 1988; McCarty and Vath, 1962), although the specific required vitamins and/or amino-acids are often unknown in these processes. In these cases the use of yeast extract (YE) is advised, since this provides a cocktail of the necessary vitamins, amino-acids and trace elements for bacteria and methanogens (Gonzalez-Gil et al., 2003). It is proposed to use a YE addition of 0.05 $gYE/gCOD_{bio\text{-}influent}$. This can be specifically fine-tuned for each case. E.g. in cases where the requirements of the organism are known, it is advised to use the defined medium generally used to culture this specific organism. For example, lactic acid bacteria have exact nutritional requirements. The yeast extract (YE) requirement is about 0.15 $gYE/gCOD_{bio\text{-}influent}$ for lactic acid production without cell recycle, but only 0.04 $gYE/gCOD_{bio\text{-}influent}$ with cell recycle (Oh et al., 2003; Pritchard and Coolbear, 1993). *Clostridium kluyveri* achieves maximum growth rate at a YE concentration of about 0.5 gYE/l, if acetate and ethanol are not limiting (Bornstein and Barker, 1948). In these cases the yeast extract dosing should be adjusted to the organism aimed for.

7 Bio-availability of trace elements

Trace element bio-availability in fermentation systems can be low because of the formation of precipitates. The solubility of trace metals is, in many cases, mainly determined by the concentrations of sulphide, carbonate and phosphate and to a lesser extent by hydroxide (Callander and Barford, 1983a). Making a nutrient solution by mixing various compounds can lead to the loss of bio-availability of trace elements because they form new salts with lower aqueous solubility compared to the original constituent chemical compounds. This possibly leads to undesired precipitation of the elements (Allwood and Kearney, 1998; Gonzalez-Gil et al., 1999). Care should be taken when combining nutrients like trace elements and phosphates in concentrated media, which can form precipitates. Addition of chelating agents results in large reductions in the free element ion concentrations, but can still keep them available for the microbial population (Callander and Barford, 1983a, 1983b). If the chelator concentration and stability constants are high enough, the free element ion concentration can be reduced to concentrations below the solubility products of sulphide, carbonate and phosphate, thereby preventing or inhibiting precipitation. Chelating agents also reduce the potential toxicity of soluble trace elements by chelating them (Agrawal et al., 2011; Milanovich et al., 1975). The most frequently used chelators are citric acid, Nitrilotriacetic acid (NTA) and

Ethylenediaminetetraacetic acid (EDTA), which are discussed separately below. Care must be taken when selecting which chelating agent to use because these agents can make trace elements more or less bio-available depending on the conditions. If the microorganism has an element binding agent that is stronger than the chelating agent, the element will be more bio-available, if not, it will be less bio-available. Callander and Barford (1983) proposed a method to calculate the soluble elements' ion concentrations. This proposed method works for defined media. However, with a complex medium (which contains (undefined) natural chelating compounds) there can be a significant discrepancy between the calculated level of free element ions and the measured amount of free element ions (Callander and Barford, 1983b). Therefore, it seems that organic ligands from microbial or vegetable origins; such as amino-acids, polypeptides and humic acids, probably play a major role in the bio-availability of trace elements (Callander and Barford, 1983b; Sillén et al., 1964; Speece, 1996). It is possible that over time an anaerobic process becomes able to generate, in sufficient amounts, the necessary chelators that make trace elements bio-available (Speece, 1996). Chelators produced by in situ microorganisms could be dependent on the specific sludge load and type of substrate that is used. Additional trace element supplementation has been shown to be beneficial when changing the substrate or during non-steady state conditions; such as a start-up, change in loading rate, or change of substrate (Henry et al., 1996). At higher temperatures the solubility product constant of carbonate and phosphate metal salts is in many cases lower (Braun, 1991; Friedfeld et al., 1998). Therefore, freely available metal concentrations are generally lower in thermophilic fermentation or anaerobic digestion systems compared to mesophilic systems, increasing the importance of (natural) chelators at elevated temperatures.

As mentioned above, the most commonly used chelators are citric acid, NTA and EDTA. These chelators require at least two of the carboxylic acid groups to be dissociated before they can act as chelators (Rivers and Umney, 2003). For this reason, the different pKa values of citric acid, NTA and EDTA are provided in table 9.

Each chelator has its advantages and disadvantages. An overview of the advantages and disadvantages of these three chelators is given in the following sections.

Citric acid

Citric acid is a chelator which is very soluble (Dalman, 1937). To guarantee two dissociated carboxylic acid groups, the pH must be above 4.76, according to table 9. However, a great drawback of the use of citric acid, is that it can be (an)aerobically degraded (Aquino and Stuckey, 2007; Veeken, 1999).

NTA

Nitrilotriacetic acid (NTA) is a chelator that is hardly soluble in its acidic form. To increase the solubility of NTA and to let NTA chelate trace elements, the pH has to be increased to 6 or higher (Rivers and Umney, 2003). However, the pH shouldn't be too high because then the trace elements could precipitate as hydroxides over time (Rivers and Umney, 2003). Free NTA does not interfere with anaerobic digestion (Aquino and Stuckey, 2007). Although NTA is quite resistant to biodegradation it can be degraded given enough time (Nörtemann, 2005).

EDTA

EDTA increases the bio-availability of trace elements. Addition of EDTA causes a reduction in the amount of trace elements that needs to be added (Vintiloiu et al., 2013) and also does not affect the activity of enzymes (Mahadevan et al., 1977). A drawback is that free EDTA can reduce the methanogenic production rate. It is suggested that this is because EDTA can act as a stronger chelator than the chelating sites on the cell surface (Aquino and Stuckey, 2007). EDTA can also chelate bivalent cations like Ca^{2+} and Mg^{2+} which could possibly result in the reduction of biofilm and granule stability (Grotenhuis et al., 1991). Therefore, care should be taken to ensure that most EDTA is in its chelated form.

Because of the degradability of citric acid, and the possibility of excessive chelation of trace elements and bivalent cations by EDTA, NTA is the preferred chelator to use in our opinion.

8 Toxicity

Trace elements are necessary, but at high concentrations they can become inhibitory or even toxic. (Nandan et al., 1990). Inhibition and/or toxicity is often caused by the chemical binding of trace elements to enzymes, resulting in disruption of the enzyme structure and consequently, inactivation of the enzyme itself (Vallee and Ulmer, 1972). The toxicity of trace elements depends on their chemical forms and their dissolved concentrations (Lin, 1993). Trace element toxicity can be reduced or prevented by chelation (Aquino and Stuckey, 2007; Babich and Stotzky, 1983). In flocculated or granular form, methanogens in anaerobic sludge show higher resistance to trace element toxicity in comparison to singe cell cultures (Lin and Chen, 1997; Pedersen and Sayler, 1981). However, adaptation by the biomass culture to higher trace element concentrations can also occur over time (Chen et al., 2008). This may be the result of internal changes in the predominant species, or of a shift in the population (Chen et al., 2008).

Of the microorganisms involved in the anaerobic digestion process, methanogens are generally considered to be the most sensitive members of the anaerobic consortia to trace element toxicity (Feng et al., 2010) Acidogens are thought to be more resistant than methanogens (Zayed and Winter, 2000). In tables 10 and 11 an overview is provided of the literature resourced inhibitory concentrations for different trace elements for mesophilic acidogens and mesophilic methanogens. The inhibitory concentrations of trace elements for thermophilic systems is not presented due to a lack of information. For practical purposes, the inhibitory/toxic trace element concentration values for mesophilic systems can also be used for thermophilic systems.

For both mesophilic acidogens and methanogens the trace element concentration at which inhibition started, as well as the IC_{50} concentrations, were sourced from the literature. The 'concentration at which inhibition started' means the concentration at which a reduction of maximum growth rate starts to occur.

For iron, cobalt, zinc, manganese, molybdenum, selenium, tungsten and boron no concentrations at which inhibition started, and IC_{50} concentrations for mesophilic acetogenic and methanogenic (suspended cell) cultures could be found.

If no information is present regarding the toxicity of a trace element, the value of the most inhibitory element can be used for that specific group. References indicate that the most inhibitory element for acidogens is nickel and for methanogens it is copper (Lin and Chen, 1997; Sanchez et al., 1996).

9 Typical form of addition for the elements

In table 12 an overview is given of the typical forms that are commonly used to supply the required macronutrients and trace elements.

Bacteria and Archaea require elemental sulphur. If sulphate is dosed it will be reduced to sulphide at the expense of available COD. Most organisms can use sulphide or cysteine and/or methionine to fulfil this requirement (Jarrell and Kalmokoff, 1988). However, sources of sulphur other than sulphide can result in lower growth rates (Daniels et al., 1984). Excess sulphide has however, amongst others, the disadvantage of promoting formation of H_2S or sulphide precipitates, depending on the pH. The sulphide precipitates can cause issues such as fouling of electrode surfaces. Overdosing of sulphide should thus be avoided if possible.

10 Conclusion

Until now most published results on anaerobic digestion and fermentative conversions are not comparable because of the different nutrient media used. This review paper is an attempt to standardize the composition of nutrient media for growing anaerobic consortia. After an extensive review of current literature, it can be concluded that the required amount of macronutrients should be based on the biomass growth, while the required amount of trace metals should be based on the biodegradable COD concentration in the influent. A distinction can be made regarding nutrient requirements between anaerobic digestion and fermentative processes without the aceticlastic/methanogenic phase. It should be noted that thermophilic bacteria and archaea have a trace metal requirement, which is possibly a factor of 2 to 5 times higher than for mesophilic bacteria and archaea.

For stable operation of an anaerobic digester, an analysis should be made of the trace metals present in the organic waste(water) stream that will be treated to judge if additional trace metal dosage is required. If the substrate of a stable running digester is changed, the present microbial population has to adapt to this new substrate. During the adaptation period it is advised to add trace metals and vitamins during this adaptation period, where after this dosage could be slowly reduced The same applies for fermentation, although addition of vitamins and amino-acids could lead to a change in fermentation product spectrum.

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Conflict of Interest statement

The authors report no conflict of interest.

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1137 **Table 1** **Macronutrients (adapted from** Kayhanian and Rich (1995)**)**

Macronutrients		
Element	**Roles**	**Reference**
C	Carbon is a basic building block of bacterial cell material. If a carbon-containing compound is oxidized or reduced, the reaction generates energy. Because organic substrates are carbon-rich, carbon will generally not be a limiting nutrient.	(Takashima et al., 1990; Wang et al., 1979)
N	Nitrogen is the primary nutrient required for microbial synthesis of proteins.	(Takashima et al., 1990; Wang et al., 1979)
P	Phosphorus is amongst others involved in the synthesis of nucleic acids and of the energy carrier ADP/ATP	(Takashima et al., 1990; Wang et al., 1979)
K	Potassium is involved in the active transport of compounds across the bacterial membrane, e.g. the transport of nutrients and providing cation balancing.	(Takashima et al., 1990; Wang et al., 1979)
Na	Counter ions to cellular buffers, electrolytes and DNA, solutes for exchange and transport processes, energy generation	(Maret and Wedd, 2014)
S	Building block of cell materials, amino acids, and of numerous enzymes	(Bryant et al., 1971; Speece and McCarty, 1964; Wolfe, 1971; Zehnder and Wuhrmann, 1977)
Ca	Required for stability of methyltransferase and aggregation of cells, also required for stability and activity of cellulases, amylases, lipases and proteases	(Bertini and Luchinat, 1994; Brahmachari et al., 2017; Gupta et al., 2004; Holmes and Matthews, 1981; Latt et al., 1969; Lytle et al., 2000; Jisha et al., 2013; Rao et al., 1998; Schwarz, 2001; Van der Meijden et al., 1984)
Mg	Required for stability of methyltransferase, stimulate methyl-CoM reductase, methenyl-H_4MPT cyclohydrolase	(Inatomi, 1986; Kenealy and Zeikus, 1982; Nelson and Ferry, 1984)

1138

1139 **Table 2 Micronutrients: elements in enzymes (adapted from Zandvoort et al. (2006))**

Enzyme	Element	References
Superoxide dismutase	Cu, Fe, Zn, Mn	(Adams et al., 1986; Hughes and Poole, 1991; Kirby et al., 1981)
Hydrogenase (facultative anaerobes)	Cu	(Adams et al., 1986; Patel et al., 1993)
NO_x reductase	Cu, Fe, Mo	(Ferguson, 1994; Schindelin et al., 1997)
Ammoniummonooxygenase	Cu	(Ensign et al., 1993)
Acetyl-CoA synthase	Cu, Ni, Fe	(Hausinger, 1987; Mulrooney and Hausinger, 2003; Seravalli et al., 2003; Thauer et al., 1980)
CO-dehydrogenase	Ni, Co, Fe	(Ferry, 1999; Schönheit et al., 1979b)
Methyl-CoM reductase (F_{430})	Ni, Zn	(Hausinger, 1994, 1987; Sauer and Thauer, 2000)
Urease	Ni	(Hausinger, 1994, 1986)
Stabilize DNA, RNA	Ni	(Hausinger, 1987; Thauer et al., 1980)
B_{12}-enzymes, vitamin B_{12}	Co	(Beveridge and Doyle, 1989; Hendlin and Ruger, 1950)
present in corrinoids	Co	(Schönheit et al., 1979b)
Tetrachloroethene reductive dehalogenase	Co, Fe	(Neumann et al., 1996)
Methyltransferase	Co, Mn	(Beveridge and Doyle, 1989; Fisher et al., 1973; Perry and Silver, 1982)
Hydrogenase	Fe, Se, Zn, Ni	(Albracht, 1994; Brock, T. D. et al., 1984; Fauque et al., 1988; Hausinger, 1987; Jones and Stadtman, 1977; Patel et al., 1993; Sawers, 1994; Stadtman, 1980; Takashima et al., 1990)
Glycin reductase	Se	(Heider and Bock, 1993)
Formate dehydrogenase	Se, Fe, Zn, Mo, W	(Adams et al., 1986; Brock, T. D. et al., 1984; GÃ-rio et al., 1992; Kirby et al., 1981; Sawers, 1994; Schauer and Ferry, 1982)
Formylmethanofuran-dehydrogenase	W or Mo	(Bertram and Thauer, 1994; GÃ-rio et al., 1992; Zellner and Winter, 1987)
Aldehyde-oxydoreductase, essential in acetogenic Clostridia	W or Mo	(White and Simon, 1992)
Essential in cytochrome	Fe	(Neumann et al., 1996)
Methane monooxygenase	Fe	(Lipscomb, 1994)
Nitrogenase	Fe	(Schindelin et al., 1997)
Proteases	Zi, Co, Mn	(Bertini and Luchinat, 1994; Holmes and Matthews, 1981; Latt et al., 1969; Jisha et al., 2013; Rao et al., 1998)

1140

1141 **Table 3. General mesophilic and thermophilic biomass yields for the different anaerobic phases**

Phase	Mesophilic conditions		Thermophilic conditions	
	Yield ($gVSS/g\ COD_{bio}$)	**Reference**	**Yield ($gVSS/g\ COD_{bio}$)**	**Reference**
Hydrolysis and Acidogenic	≈0.15-0.18	(de Kok et al., 2013; Henze and Harremoës, 1983; Karadag and Puhakka, 2010; Silley and Armstrong, 1984; Wiegant, 1986)	≈0.1	(Oh et al., 2004)
Acetogenic and methanogenic	≈0.03-0.04	(Henze and Harremoës, 1983; Takashima and Speece, 1989; Zoetemeyer et al., 1982)	0.02-0.05	(Ahring and Westermann, 1985a; Clarens and Molleta, 1990)
Hydrolysis, acidogenic, acetogenic and methanogenic combined	≈0.18 – 0.22	(Borja et al., 1995; Henze and Harremoës, 1983; Pavlostathis and Giraldo-Gomez, 1991; Rittmann and McCarty, 2001; Takashima et al., 2011; Wolin et al., 1963)	≈0.06-0.09	(Borja et al., 1995; Takashima et al., 2011)

1142

1143 **Table 4 Elemental composition biomass regarding the macronutrients from literature and proposed value to calculate required**
1144 **amount of macronutrients**

Element	Unit	Value		References	Proposed value
		Minimum	Maximum		
C	mg/gVSS	315	374	(Scherer et al., 1983; Takashima and Speece, 1989; Tchobanoglous et al., 2003; Whitman et al., 1982)	-
N	mg/gVSS	80.8	108.8		109
P	mg/gVSS	4.3	23.8		24
K	mg/gVSS	1.1	42.5		42
Na	mg/gVSS	3	40.0		40
S	mg/gVSS	5.6	12		12
Ca	mg/gVSS	0.078	3.8		3.8
Mg	mg/gVSS	0.077	0.45		0.45

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1146 **Table 5. Proposed trace element requirement for mesophilic acidogenic systems**

Trace element	Range in literature	References	Proposed value
	µg/g $COD_{bio\text{-}influent}$		µg/g $COD_{bio\text{-}influent}$
Iron	112-167	Qiang et al., 2012; Schönheit et al., 1979a; Takashima et al., 2011	165
Cobalt	0.7-14.2	Banks et al., 2012; Qiang et al., 2012; Takashima et al., 2011	14
Nickel	2.3-4.8	Qiang et al., 2012; Takashima et al., 2011	5
Zinc	29	Takashima et al., 2011	29
Copper	-	-	5
Manganese	-	-	3.75
Molybdenum	-	-	10.5
Selenium	-	-	48
Tungsten	-	-	1.65
Boron	-	-	0.057

1147

1148 **Table 6. Proposed trace element requirements for mesophilic acetogenic/methanogic systems**

Trace element	Range in literature	References	Proposed value	
	µg/g $COD_{bio\text{-}influent}$		µg/g $COD_{bio\text{-}influent}$	
Iron	21-84	Qiang et al., 2012; Scherer et al., 1983; Takashima and Speece, 1989; Weimer and Zeikus, 1978; Whitman et al., 1982; Zhang et al., 2003	84	
Cobalt	3.6-25	Florencio et al., 1994; Koster and Lettinga, 1984; Qiang et al., 2012; Scherer et al., 1983; Takashima and Speece, 1989; Weimer and Zeikus, 1978; Whitman et al., 1982; Zhang et al., 2003	5	25*
Nickel	0.9-5.4	(Scherer et al., 1983; Takashima and Speece, 1989)	5.4	
Zinc	0.7-18.9	Scherer et al., 1983; Takashima and Speece, 1989; Whitman et al., 1982; Zhang et al., 2003	19	
Copper	5-6.1	Scherer et al., 1983; Zhang et al., 2003	5	
Manganese	0.05-0.75	Scherer et al., 1983; Whitman et al., 1982	0.75	
Molybdenum	2.1	Scherer et al., 1983	2.1	
Selenium	9.6	Whitman et al., 1982	9.6	
Tungsten	0.02-0.33	Zellner and Winter, 1987	0.33	
Boron	0.0114	Whitman et al., 1982	0.0114	

1149 * The higher value of 25 µg/g CODbio-influent should only be used in the case of direct conversion of methanol into methane
1150 (Florencio et al., 1994)

1151 **Table 7. Proposed trace element requirement for thermophilic acidogenic systems**

Trace element	Range in literature	References	Proposed value
	µg/g $COD_{bio\text{-}influent}$		µg/g $COD_{bio\text{-}influent}$
Iron	230-840	Nishio et al., 1991; Qiang et al., 2013; Takashima et al., 2011	840
Cobalt	4.1-45	Qiang et al., 2013; Takashima et al., 2011	45
Nickel	1.8-19.6	Qiang et al., 2013; Takashima et al., 2011	19.6
Zinc	144	Takashima et al., 2011	144
Copper	-	-	5
Manganese	-	-	11.25
Molybdenum	-	-	31.5
Selenium	-	-	144
Tungsten	-	-	4.95
Boron	-	-	0.057

1152

1153 **Table 8. Proposed trace element requirements for thermophilic acetogenic/methanogic systems**

Trace element	Range in literature	References	Proposed value
	µg/ g $COD_{bio-influent}$		µg/ g $COD_{bio-influent}$
Iron	24.6-280	Duboc et al., 1995; Nishio et al., 1991; Qiang et al., 2013; Takashima et al., 2011	280
Cobalt	0.5-9	Duboc et al., 1995; Qiang et al., 2013; Schönheit et al., 1979b; Takashima et al., 2011	9
Nickel	27-29	Duboc et al., 1995; Qiang et al., 2013; Takashima et al., 2011	29
Zinc	1.8-96	Duboc et al., 1995; Takashima et al., 2011	96
Copper	-	-	5
Manganese	-	-	0.75
Molybdenum	-	-	6.3
Selenium	-	-	28.8
Tungsten	-	-	1
Boron	-	-	0.01

1154

1155 **Table 9. pKa values of citric acid, NTA and EDTA (Rivers and Umney, 2003)**

Chelating agent	**pKa**
Citric acid	3.13
	4.76
	6.4
NTA	1.9
	2.5
	9.8
EDTA	2.0
	2.7
	6.2
	10.3

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1157 **Table 10 Inhibitory concentrations trace elements at which inhibition started and IC_{50} values for mesophilic acidogenic cultures**

Trace element	Range in literature (mg/l)		References	
	Start inhibition	IC_{50}	Start inhibition	IC_{50}
Iron	50- >100	-	Hubbert et al., 1958; Martinez and Church, 1970	-
Cobalt	0.6-7	-	Hubbert et al., 1958; Martinez and Church, 1970	-
Nickel	0.1-0.5	100 – 1300	Martinez and Church, 1970	(Forsberg, 1978; Li and Fang, 2007; Lin, 1993; Zheng and Yu, 2004)
Zinc	3.5-20	3.4 – 1500	Hubbert et al., 1958; Lin, 1993; Martinez and Church, 1970	(Forsberg, 1978; Li and Fang, 2007; Lin, 1993; Zheng and Yu, 2004)
Copper	0.5-1.0	0.7 – 350	Hubbert et al., 1958; Martinez and Church, 1970	(Forsberg, 1978; Li and Fang, 2007; Lin, 1993; Zheng and Yu, 2004)
Manganese	50-100	-	Martinez and Church, 1970	-
Molybdenum	500	-	Martinez and Church, 1970	-
Selenium	5	0.038 – 0.14	Martinez and Church, 1970	(Forsberg, 1978)
Boron	200	-	Martinez and Church, 1970	-

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1159

1160 **Table 11. Inhibitory concentrations trace elements at which inhibition started and IC_{50} values for mesophilic**
1161 **acetogenic/methanogenic cultures**

Trace element	Range in literature (mg/l)		References	
	Start inhibition	IC_{50}	Start inhibition	IC_{50}
Nickel	1-5	1 – 1600	Ahring and Westermann, 1985b	(Ahring and Westermann, 1985b; Altaş, 2009; Fang and Chan, 1997; Fang and Hui, 1994, 1994; Lin and Chen, 1999, 1997; Lin, 1992, 1992)
Zinc	-	7.3 - 270	-	(Altaş, 2009; Fang and Chan, 1997; Fang and Hui, 1994, 1994; Lin and Chen, 1999, 1997; Lin, 1992, 1992)
Copper	1-10	1 - 175	Ahring and Westermann, 1985b	(Ahring and Westermann, 1985b; Altaş, 2009; Fang and Chan, 1997; Fang and Hui, 1994, 1994; Lin and Chen, 1999, 1997; Lin, 1992, 1992)

1162

1163 **Table 12. Typical form of addition of elements (adapted from** krishna Ravuri (2013)

Element	**Typical form of addition**
Macronutrients	
Nitrogen	NH_3, NH_4Cl, NH_4HCO_3
Phosphorus	NaH_2PO_4
Potassium	KCl
Sodium	NaCl, $NaHCO_3$
Sulfur	Na_2S, cysteine, -SO_4 salt
Calcium	$CaCl_2$
Magnesium	$MgCl_2$, $MgSO_4$
Trace elements	
Iron	$FeCl_2$, $FeSO_4$
Cobalt	$CoCl_2$
Nickel	$NiCl_2$
Zinc	$ZnCl_2$, $ZnSO_4$
Copper	$CuCl_2 \cdot 2\ H_2O$, $CuSO_4$
Manganese	$MnCl_2$
Molybdenum	$NaMoO_4$
Selenium	Na_2SeO_3
Tungsten	$NaWO_4$
Boron	H_3BO_3

1164